



Interferon Response Detection Kit

for validation of siRNA experiments

(Cat. # SI300A-1)

User Manual

Store kit at -20°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

(ver. 6-020112)

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I. Introduction and Background

A. Overview

This manual provides details and information necessary to use the Interferon Response Detection Kit to identify and reproducibly confirm non-specific stress response of cells to the introduction of siRNA. This kit may also be used for other applications, such as for the detection of retroviral infection. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this system.

B. siRNA and Interferon Induction

Mammalian hosts have evolved defense mechanisms against double-stranded RNA (dsRNA), typically present as a consequence of viral infection. Upon viral infection, cytokine interferon is induced. Interferon then activates a signaling cascade, culminating in transcriptional activation of hundreds of interferon stimulated genes (ISGs). Many of these ISGs function in stress response pathways to mediate the cellular antiviral response (Haque and Williams, 1998; Stark *et al.*, 1998).

The use of siRNA to selectively knockdown genes of interest has become a powerful tool to study gene function. Recently, it was discovered that, in addition to gene-specific silencing, siRNA can also induce nonspecific effects in cells by activating ISGs involved in the stress response (Bridge *et al.*, 2003; Persengiev *et al.*, 2004; Scacheri *et al.*, 2004; Sledz *et al.*, 2003; Pebernard, 2004). The observation that treatment of mammalian cells with siRNA molecules can trigger non-specific cellular responses raises a serious concern about specificity of RNA interference responses and complicates the interpretation of siRNA knockdown results. To address this issue, new tools are needed that help confirm the specificity of responses to the knockdown of genes targeted by experimental siRNA and the absence of off-target effects induced by the presence of the siRNA molecules.

SBI's Interferon Response Detection Kit provides an easy and accurate method to ensure that synthetic siRNA or siRNA constructs in plasmid or viral vectors do not induce significant interferon-related responses when expressed in the cell system of choice. Also, the system can be used to assess the effects of reagents (such as transfection reagents) and other procedural steps that may produce, enhance, or otherwise affect cellular interferon responses.

While not all siRNA molecules will activate non-specific cellular stress response pathways, the only way to confirm the specificity of an siRNA knockdown phenotype or response is to also confirm the

absence of any nonspecific cellular effects. SBI's Interferon Response Detection Kit provides an objective and unambiguous procedure to ensure non-specific activation of interferon-related pathways do not perturb your siRNA knockdown studies.

C. Detection of Interferon Pathway Activation

SBI's Interferon Response Detection Kit uses a PCR-based approach to enable researchers to easily measure relative expression levels of five genes involved in the interferon response. The use of these five genes helps ensure the kit will accurately detect interferon-related stress responses in a variety of cell types and under many different conditions. Different cell types may show different levels of increase for these genes. Also, the level of interferon-related stress response that is observed will vary depending on the concentration of the siRNA molecule (Persengiev *et al.*, 2004) and the cell line.

The OAS1 (NM_016816) and OAS2 (NM_016817.1) genes:

The 2',5'-oligoadenylate synthetases (OAS) represent a family of interferon-induced proteins implicated in the mechanism of the antiviral action of interferon. When activated by double-stranded RNA, these proteins polymerize ATP into 2'-5'-linked oligomers that bind and activate RNase L, which plays a significant role in the inhibition of cellular protein synthesis and viral infection resistance.

Three forms of human OAS have been described (Hovnanian A, *et al.*, 1998). This kit contains primers to detect two of the three forms: OAS1 and OAS2. There are two isoforms of the OAS1 gene which share identical N-terminal sequence but diverge at exon 7 and have molecular weights of 40 and 46 KDa. The Primer Mixture for OAS1 amplifies a 144 base region present in both isoforms. Similarly, there are two isoforms of OAS2 gene which also share a common 5' region and have molecular weights of 69 and 71KDa. The OAS2 Primer Mixture amplifies a 234 base region present in both isoforms.

The MX1 (NM_002462.2) gene:

Human MX1 protein is a member of the interferon-induced myxovirus (influenza virus) resistance protein family (MX proteins) and an important component of the innate host defense against RNA viruses. The MX family belongs to a superfamily of large GTPases that also includes the dynamins and the interferon-regulated guanylate-binding proteins. MX1, by interacting with a component of the nucleocapsid, prevents replication of viral RNA and thereby inhibits the production of new infectious virus particles. (Kochs G, *et al.*, 2002). The MX1 Primer Mixture amplifies a 402 nucleotide region of this gene.

The IFITM1 (NM_003641.2) gene:

Interferon-inducible trans-membrane proteins (IFITMs) of approximately 17 kDa have been suggested to play a role in the

antiproliferative activity of interferons based on their pattern of induction in interferon-sensitive and interferon-resistant cell lines and their ability to inhibit cell growth when a membrane fraction enriched in 17-kDa proteins is added to cells in culture. IFITM1 has been demonstratively shown to associate with other proteins at the cell surface to form a complex relaying growth inhibitory and aggregation signals. (Deblandre G.A, *et al.*, 1995). The IFITM1 Primer Mixture amplifies a 128 base fragment of this gene.

The ISGF3 γ (NM_006084.3) gene:

Alpha interferon stimulates transcription by converting the positive transcriptional regulator ISGF3 from a latent to an active form. This receptor-mediated event occurs in the cytoplasm, with subsequent translocation of the activated factor to the nucleus. ISGF3 has two components, termed ISGF3 α and ISGF3 γ . ISGF3 γ serves as the DNA recognition subunit, while ISGF3 α is the target for interferon signaling and serves as a regulatory component. (Veals S.A., *et al.*, 1992). The ISGF3 γ Primer Mixture amplifies a 333 base portion of the gene.

D. Variability of the Interferon Response

The sensitivity of different cell lines to double-stranded RNA activation of interferon-induced stress genes and pathways can vary significantly. Cell type, as well as growth conditions and passage number, can affect the susceptibility, level, and extent of activation of the interferon response. The interferon response is readily detected in the numerous human cell lines that activate stress response pathways when double-stranded RNA is introduced. This response can be clearly seen by the robust increase in OAS1, OAS2, MX1, ISGF3 γ and IFITM1 expression levels after treatment with concentrations as low as 250 ng/ml of a long poly(I)-poly(C) dsRNA sequence (Sigma, Cat. # P9582) in cell lines such as HEK 293 and MRC-5 (see Figure 1). Higher concentrations of the double-stranded RNA (*e.g.* 2.5 μ g/ml) can trigger rapid apoptosis and cytotoxicity within 24 to 48 hours.

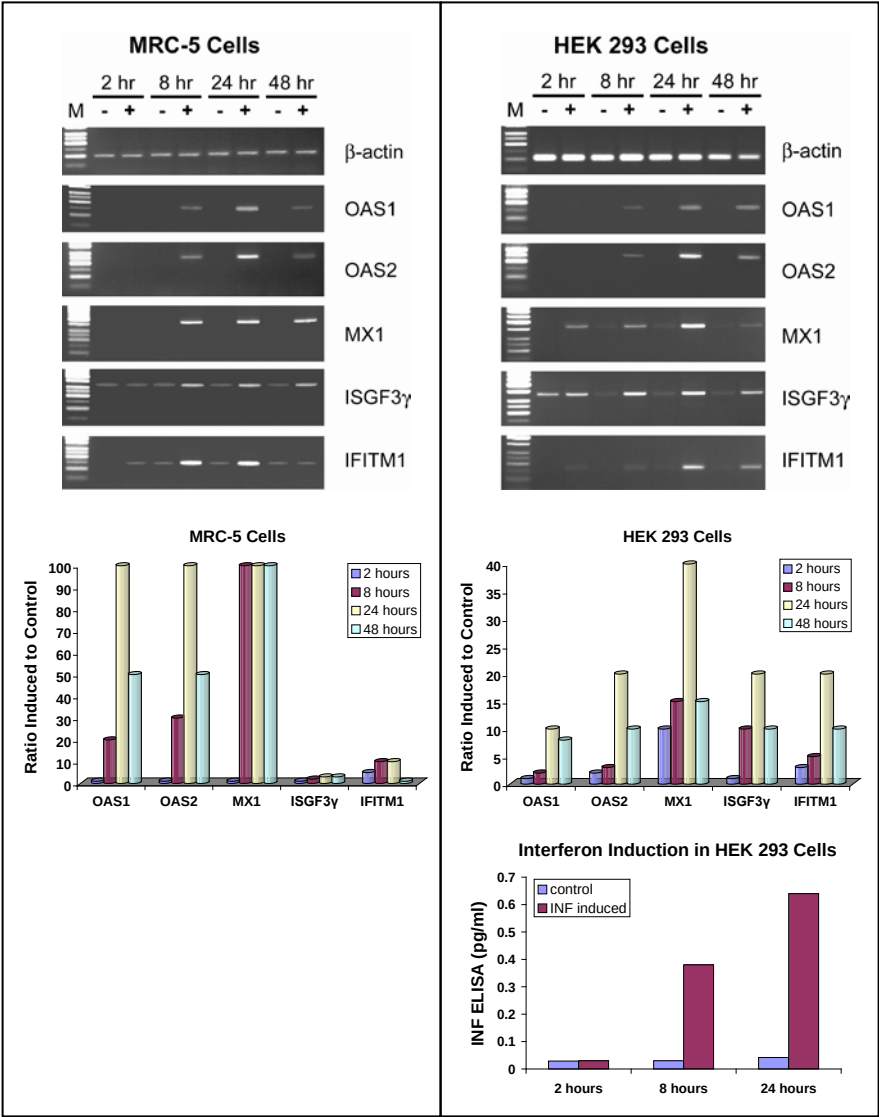


Fig. 1. Interferon response detected in two human cell lines. MRC-5 and HEK 293 cells were grown to confluency in a T-75 format flask, and then transfected with 500 ng/ml of a long poly(I)-poly(C) dsRNA sequence (Sigma, Cat. # P9582). Total RNA was prepared from treated (+) and mock transfected (-) control cells at 2, 8, 24, and 48 hours after transfection. The expression levels of OAS1, OAS2, MX1, ISGF3 γ and IFITM1 genes were determined by end-point RT-PCR (27 cycles) using the protocol described in the kit. As is evident from the results shown for MRC-5 cells (on the left) and HEK293 cells (on the right), the interferon response was readily detected by examination of the expression levels of these genes. Also shown is the level of interferon response in HEK 293 cells as detected by ELISA.

E. Overview of Protocol

As can be seen in the flowchart in Figure 2, the procedure for the Interferon Response Detection Kit is straightforward, convenient, and provides clear, measurable results.

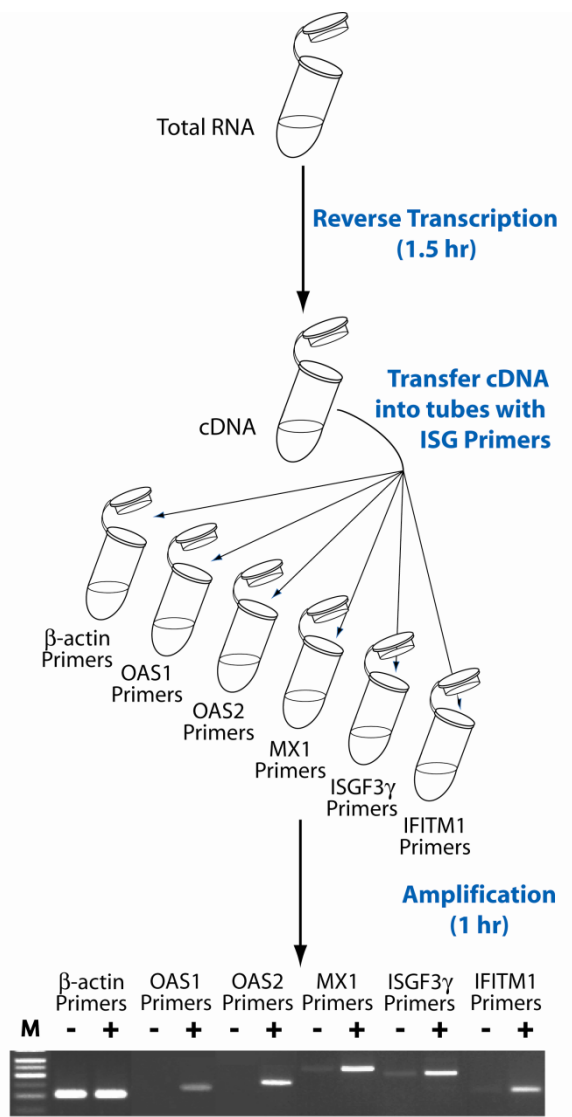


Fig. 2. Flowchart for IRD Kit Procedure.

F. List of Components

The Interferon Detection Kit provides enough primer, in each mix, to perform 20 PCR reactions (100 total reactions) in 50 μ l.

20 μ l	Primer Mixture for OAS1	144 nt*	10 μ M ea.
20 μ l	Primer Mixture for OAS2	234 nt	10 μ M ea.
20 μ l	Primer Mixture for MX1	402 nt	10 μ M ea.
20 μ l	Primer Mixture for IFITM1	128 nt	10 μ M ea.
20 μ l	Primer Mixture for ISGF3 γ	333 nt	10 μ M ea.
20 μ l	Control Primer Mixture for β -actin	110 nt	10 μ M ea.
15 μ l	-ve Control cDNA (uninduced 293TN cells)		0.4mg/ml
15 μ l	+ve Control cDNA (PolyI/PolyC induced 293TN cells)		0.4mg/ml

**indicates size of the amplicon.*

The kits are shipped on blue ice and should be stored at -20°C upon receipt. Properly stored kits are stable for 1 year from the date received.

G. Additional Required Materials

- RNeasy Mini Total RNA Purification Kit (QIAGEN, Cat. # 74104)
- Reverse Transcription Kit (SuperScript™ III, Invitrogen, Cat. # 18080-051) or, for small quantities of RNA, use SBI's Full Spectrum™ Complete Transcriptome RNA Amplification Kit (Cat. # RA101A-1)
- PCR Reagents (Titanium™ Taq DNA Polymerase, Clontech, Cat. # 639208)
- Thermocycler (with heated lid)
- 3% Agarose Gel in Tris-Acetate EDTA (TAE) Buffer
- DNA Size Ladder with markers from 50 to 2,000 bp (AmpliSize™ DNA Ladder, Bio-Rad, Cat. # 170-8200)
- Optional for samples from sources with high RNase activity: Ribonuclease Inhibitor (SUPERase-IN™, Ambion, Cat. # 2694)

H. Procedural Guidelines

Basal and induced levels of OAS1, OAS2, MX1, ISGF3 γ , and IFITM1 may vary from human cell line to human cell line. In addition, basal and induced levels of the ISGs may also vary in the same cell line depending on growth conditions. Thus, it is critical to ensure that you

include a non-induced control that is treated exactly the same as samples where you introduce siRNA—this includes mock transfecting the control cells.

Since siRNA reagents are delivered to cells via transfection, it is crucial to consider the transfection efficiency of the cells when assessing the sensitivity of a particular cell line to the interferon-related response to siRNA.

In addition to the specific procedural notes above, be sure to follow these general practices to ensure quality results:

- Before dispensing, completely thaw all reagents. Vortex, to mix thoroughly, all reagents except for the enzymes. After adding reagents to the mixture, pipette up and down 5-10 times to ensure mixing.
- Briefly centrifuge each mixture once all the components have been added to ensure there are no reagents left on the sides of the tube, separated from the reaction mixture.
- When setting up multiple reactions, we recommend that you prepare a master mix.
- cDNA is relatively stable and can be stored for a few hours at room temperature or 4°C. For longer storage, place at -20°C.
- It is important to perform the amplification with the control cDNA provided with this kit. The amplified products generated with this control cDNA should be saved and used in subsequent experiments to verify correct amplicon sizes. Without this control reaction, it will be difficult to troubleshoot any unexpected results.

II. Protocol

A. Isolating RNA

In general, you can use any procedure that provides you with high quality total RNA. In our labs, the QIAGEN RNeasy Total RNA Purification Kit (Cat. # 74104) provides consistent and reproducible yields.

If possible, confirm the quality of your RNA before starting the amplification. The Agilent BioAnalyzer offers a convenient, sensitive, and reliable method to test small amounts of RNA. Alternatively, you can run the RNA on an agarose gel to make sure it is intact.

The average expected yields of total RNA for 1×10^6 cells of some common cell types is listed below:

<u>Source</u>	<u>µg total RNA</u>
NIH/3T3	10
HeLa	15
COS-7	35
LMH	12
Huh	15

We recommend that you collect your cells at approximately 24 hours after you transfect or after the addition of components that you want to test for interferon induction. Although you can often begin to see the increase in some genes in as few as 8 hours (see Figure 1), it is advisable to wait 12 to 24 hours to ensure that you get maximal induction of the interferon response marker genes.

For this procedure, start with 1 µg of total RNA at a concentration greater than 0.5 µg/µl. If the starting amount is significantly less than this, we recommend using SBI's Full Spectrum™ Complete Transcriptome RNA Amplification Kit (Cat. # RA101A-1) to generate enough template for gene specific PCR analysis (Part C of this protocol).

B. First-Strand cDNA Synthesis

1. For each RNA sample, set up a first-strand synthesis reaction by adding the following components to a 0.2 or 0.5 ml PCR tube:

1.0 µl	Random Primer (10 µM, not supplied)
0.5-2 µl	1 µg Total RNA

Note: Because reagent volumes are small, accurate pipetting is critical.

2. Add sufficient RNase-free water to bring the reaction volume up to 3 μ l.
3. Anneal the primers by placing the tubes in a thermocycler with a heated lid, and incubate the reactions at 70°C for 2 minutes. Then place the reactions at 25°C for 10 minutes.
4. While the reactions are incubating, set up a Synthesis Master Mix sufficient for the number of first-strand synthesis reactions you are processing. This is done by adding to a microfuge tube the volume of each of the following components multiplied by the number of reactions you are processing for each first strand reaction mix:

Synthesis Master Mix:

3.0 μ l	RNase-free Water
2.0 μ l	5X Reverse Transcriptase Buffer
1.0 μ l	10 mM dNTP Mix
0.5 μ l	100 mM Dithiothreitol (DTT)
0.5 μ l	Reverse Transcriptase (20X)
7.0 μ l	Total volume per reaction

5. After incubating each of the first-strand reactions from step 3 for 10 minutes at 25°C, add 7 μ l of the Synthesis Master Mix to each reaction tube.
6. Incubate the first-strand reactions for 1 hour at 42°C, then place them at 70°C for 4 minutes. The first-strand cDNA can be stored at -20°C until you are ready to proceed with gene-specific amplification.

C. Gene-Specific Amplification Using cDNA

This section outlines the procedure to amplify and detect the interferon marker genes using end-point PCR and agarose gel electrophoresis. Alternatively, you can use real-time PCR with SYBR Green. Details on this alternative procedure are below.

Note: For gene-specific PCR, we recommend using a “hot start” PCR polymerase.

1. For each first-strand synthesis reaction from Part B, set up 6 reactions—one for each set of gene-specific Primers (including the β -actin control Primers), by adding the following.

1.0 μ l	cDNA (from first-strand synthesis reaction)
1.0 μ l	Gene-Specific Primer Mixture

2. Set up an Amplification Master Mix by adding to a microfuge tube the volume of each of the following components multiplied by the number of first-strand synthesis reactions from Part B:

Amplification Master Mix:

30 μ l	10X PCR Buffer
6 μ l	50X dNTP Mix
6 μ l	PCR Polymerase (50X)
246 μ l	RNase-free Water
288 μ l	Total volume per first-strand synthesis reaction

3. Add 48 μ l from the Amplification Master Mix to each of the tubes contain gene-specific primers and cDNA (from Step 1).
4. Place the reactions in a thermal cycler and cycle using the following program:
 - step 1. 94°C for 3 min
 - step 2. 60°C for 1 min
 - step 3. (94°C for 30 sec; 60°C for 30 sec) for 20-30 cycles (see note)
 - step 4. 15°C hold

Note: You will need to optimize the number of cycles for step 3 of the program, depending on the amount of starting RNA. Refer to the table below to determine the approximate number of times you should cycle for samples that show a positive stress response to siRNA:

<u>Starting RNA (ng)</u>	<u># Cycles</u>
300	20
100	23
50	26
25	30

Note: Some genes may not normally be expressed—or may be expressed at very low levels—in some cell types, so you may not see a band in some control samples.

5. After amplification, run 5 μ l of each reaction on a 3% agarose gel in 1X TAE Buffer. Include a DNA size ladder with markers in the range of 50-2,000 bp (e.g., Bio-Rad AmpliSize™ DNA Ladder). You should see results similar to those shown in Figure 1.

Depending on your particular RNA sample (or cell type), more cycles may be necessary. If so, perform an additional 3 cycles and check your product again. You do not need to add additional PCR Polymerase, even if your reaction was cycled overnight, as long as you held the reaction at 15°C after cycling. You can continue adding two-cycle increments, up to 30 cycles, until you see sufficient product from your amplification reaction.

Note: Do not over cycle your PCR reactions to more than 30 cycles, because both induced and non-induced samples may result in similar band intensity or smear on the gel.

Alternative Real-Time qPCR Procedure.

Our primers can be used in real-time experiments with SYBR Green detection. For real-time qPCR we suggest TaqMan® SYBR Green.

We recommend using commercially available kits and reagents for SYBR Green qPCR, for example, Invitrogen's Platinum SYBR Green qPCR SuperMix-UDG (Cat. # 11733-038) or QIAGEN's QuantiTect SYBR Green PCR Kit (Cat. # 204143) and following the manufacturer's protocol, but increase the extension time to at least 45 seconds.

The cycling conditions we use are:

Step 1: 50°C, 2 min

Step 2: 95°C, 10 min

Step 3: (95°C --15 sec, 60°C --1 min) x 40 cycles

Step 4: (95°C --15 sec, 60°C --15 sec, 95°C --15 sec) for setting the dissociation curve

III. References

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- Veals SA *et al.*, Mol Cell Biol. 1992;12(8):3315-24.

IV. Troubleshooting

A. No Product from cDNA Amplification with Gene-Specific Primers

If you do not see bands of the expected size for any samples, including the Control cDNA and β -actin Control Primer Mix...

- One or more of the reagents were omitted during the procedure or the volume of the reactions is incorrect. Calibrate your pipette and try amplifying the Control RNA again using primers for β -actin.
- There may be a problem with your RT or PCR reagents. Replace all the reagents or use commercially available kits for RT and PCR and try to amplify with the control β -actin primers.

If you see bands for the Control cDNA, but not for your RNA samples...

- You may have less starting RNA than measured. Place the amplification reactions back in the thermocycler and perform an additional three cycles (94°C for 30 sec; 68°C for 30 sec). If, after additional cycles, there are still no bands or very weak bands compared with the Control RNA reaction, your RNA may either be (1) degraded or (2) contain an inhibitor. Try the reverse transcription again after purifying new RNA. If you still do not get sufficient yield, try a different RNA purification kit (For example Invitrogen's TRIzol Reagent, Cat. # 15596-026) or try using SBI's Full Spectrum™ Complete Transcriptome RNA Amplification Kit (Cat. # RA101A-1) to increase the amount of starting template.
- Your Reverse Transcription reaction may not have worked well. To check this, you can run the remaining portion of your reverse transcriptase reaction on a 1% agarose gel containing 0.01% ethidium bromide. You should see a smear as shown in Figure 3. If you do not see a relatively bright smear, there was a problem with your reverse transcription reaction. Do this reaction again, and check 3 μ l of the product on a gel before continuing with the amplification. If you do not see sufficient product again, contact SBI.

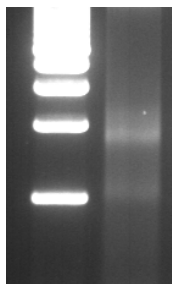


Fig. 3. Agarose gel showing product of a cDNA synthesis reaction.

If you see smears or non-specific products with your gene-specific primers...

- Confirm that you are using some form of “hot-start” PCR system that ensures specific priming.
- If you are using “hot start” and get smearing, you may be over cycling your PCR reaction, making a mistake in mixing reagents, or failing to use optimized PCR reagents.
- Reduce the number of cycles or use recommended reagents. As noted, the volume of the amplified cDNA added to the gene-specific reaction should not exceed 5%.

V. Appendix

A. Related Products

- **RNAi Cloning and Expression Lentivectors**
These FIV and HIV-based single- and double-promoter shRNA and siRNA cloning vectors allow you to clone siRNA templates and efficiently transduce these siRNA constructs in a wide range of cells. For a list of currently available vectors, please visit our website at <http://www.systembio.com>.

B. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

<http://www.systembio.com>

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VI. Licensing and Warranty Statement

Limited Use License

Use of SBI's Interferon Response Detection Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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SBI may have pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

SBI's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. SBI's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. SBI does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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